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Antifungal and toxicity effects of new combined essential oils on *Oncorhynchus mykiss* in comparison with malachite green

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Abstract

Mold infection is one of the most important problems in aquaculture. Recently, administration of some chemicals such as malachite green in order to control mold infections has been limited in aquaculture. In the current decade, researchers have been more focused on using herbal extracts and essential oils in aquaculture. In this study, Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of a new combination of four essential oils (*Eucalyptus globulus*, *Mentha piperita*, *Salvia officinalis* and *Thymus vulgaris*) were determined by serial dilution method. LC50 of the combined essential oils for 48 and 96 hours were then determined on *Oncorhynchus mykiss* fingerlings. MIC and MFC of combined essential oils were 0.025µl/ml and 0.050 µ l/ml for *F. solani* and 0.018 µ l/ml and 0.035 µ l/ml for *S. parasitica,* which was lower compared to malachite green. It was also 0.060µl/ml and 0.300 µ l/ml for *F. solani* and 0.045 µ l/ml and 0.120 µ l/ml for *S. parasitica* respectively ($p<0.05$). The results of LC50 for 48 and 96 hours were calculated equal to 34.98ppm. Based on these results, the combination usage of essential oils can be proposed as a good antifungal therapeutic strategy in hatcheries.

Keywords: mold infection, salmonid hatcheries, essential oil, MIC, MFC, LC50

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Introduction

Mold infection is one the most important problems in aquaculture. Unfertilized fish eggs are susceptible to fungal infection particularly from the family Saprolegniaceae (Post, 1987; Hussein and Hatai, 2002; Forneris *et al.*, 2003). Most disinfectants using in aquaculture, such as malachite green, are forbidden for their toxicologic, teratogenic and carcinogenic effects on fish and human (Stammati *et al*., 2005; Olesen *et al*., 2007; Sudova, 2007). In the past decade interest on the topic of antimicrobial plant extracts has been growing (Tassou *et al*., 2000; Nilsen and Rios, 2000; Yildirim *et al.*, 2000; Wong and Kitts, 2002; Valero and Salmeroj, 2003; Pinto *et al.*, 2007). Furthermore, some studies on antibacterial and antifungal activities of essential oils and herbal extracts on aquatic animal have been conducted (Marino, 2001; Bajpai *et al*., 2007; Mousavi *et al.*, 2009). However, only a few reports have studied combinations of these products for their synergistic antimicrobial activities (Lee *et al*., 2007, Mousavi *et al*., 2009). The aim of the present study was to evaluate antifungal activity and toxicity of a new herbal antifungal agent (Combined essential oils) in comparison to malachite green *in vitro* and *in vivo*.

Materials and methods

Combined essential oils

The combined essential oils (CEO) used in this study were extracted from the herbs, *Thymus vulgaris* (thyme), *Salvia officinalis* (common sage), *Eucalyptus globulus* (blue gum eucalyptus) and *Mentha piperita* (peppermint). The herbs were collected from an experimental field in the Zardband region located in the north eastern of Tehran, Iran.

Air-dried leaves and stems of the herbs (90g from each herb) were subjected to hydro distillation for 4 hours using a Clevenger-type apparatus to produce essential oils according to the method recommended by the European Pharmacopoeia (Schulz *et al*., 2004). The final combination of essential oils was prepared using an emulsifier. It was composed of 30% *Salvia officinalis*, 30% *Thymus vulgaris*, 20% *Mentha piperita* and 20% *Eucalyptus globulus* extracts. The CEO was dried over anhydrous sodium sulfate and stored in a sealed vial at low temperature (5-10º C) before analysis.

Gas Chromatography and Mass Spectrometry (GC-MS) analyses

The composition of the CEO was determined by gas chromatography (GC) and by GC coupled with mass spectrometry (MS) (Mousavi *et al*., 2009). The different components within the CEO were identified by comparisons of their mass spectra with those of a database of known spectra (Stenhagen, *et al.,* 1974) or with authenticated reference compounds (Adams, 2001). Identities were confirmed by comparison of their retention indices either with those of authenticated compounds or with data published in the literature (Stenhagen, *et al*., 1974).

Malachite Green

Malachite green (oxalate salt) was provided from Merck company branch in Tehran, Iran. This chemical is used in microbiology lab for staining and as an antifungal agent in fish hatcheries and for aquarium fishes (Mousavi *et al*., 2009).

MIC (minimum inhibitory concentration) and MFC (minimum fungicidal concentration)

Serial dilution method was used in this study (Eloff, 1998). The fungal strains (*Saprolegnia parasitica* and *Fusarium solani*) were collected randomly from infected eggs from one of the salmonid hatcheries in the north of Iran. For Sporulation, fungal specimens were cultured on glucose yeast extract agar for *Saprolegnia parasitica* and Potato dextrose agar for *Fusarium solani* and then incubated in 20 ºC for 1-2 week. After incubation, spores were collected and adjusted to 10000cell/ml.

Twenty-four sterile glass tubes were filled by ten milliliters of SDB (Sabouraud Dextrose Broth) and divided in two groups. Different serial dilutions were prepared by adding one milliliter from the stock solution of malachite green and combined essential oils to the first tube and then serially diluted to the last tube. Then, 100 µl from

spore collection solution was added to every tube and incubated for 48- 72 hours in 20 ºC. After incubation, minimum inhibitory concentrations (MICs) were determined as the lowest concentration that resulted in a complete inhibition of visible growth of the microorganisms. Minimum fungicidal concentrations (MFCs) were determined as the lowest concentration, which did not allow any visible growth of the microorganisms after subculture. This examination was performed in triplicate.

LC50 (Lethal concentration 50 per cent) 24, 48, and 96 hours on rainbow trout fingerlings

Six aquariums were filled with aired water. For adaptation, fifteen fingerlings of rainbow trout (Mean weight 3±0.2 grams) were transferred to aquaria for 96 hours.

Some physico-chemical parameters such as, temperature, PH, total hardness, dissolved oxygen, was daily estimated (table 1). After adaptation, different concentration of combined essential oils (10, 25, 50, 100, 150, 200ppm), were added to the aquaria, and any behavioral changes was recorded. After 24, 48 and 96 hours, the mortality rate was counted and recorded. This examination was performed in triplicate.

Data Analysis

The data obtained from our study was analyzed with the variance analysis and Kruskal – Wallis to compare differences between tests and controls. Mean, standard deviation and some statistical indexes was calculated and P values lower than 0.05 ($p < 0.05$) were considered to reflect significant differences among treatments.

To determine LC50, the BioStat 2008 software (probit analyses and comparing two related samples) was used.

Results

The composition of the combined essential oils from each of the herbs used to formulate the CEO was determined by GC/MS analysis (Mousavi *et al*., 2009).

The main components were 1, 8-cineol (21.37%), thymol (13.86%), camphor (7.92%), α-thujone (7.71%), menthon (6.8%) and menthol (6.2%).

The results of MICs and MFCs are presented in table 2. Based on the results, MIC of this herbal combination was 0.025 μ l/ml for *F. solani* and 0.018 μ l/ml for *S. parasitica* that was significantly different from MIC of malachite green on *F. solani* (0.060 μ l/ml) and *S. parasitica* (0.045 μ l/ml) (p<0.05).

MFC of the combined essential oils were 0.050 μ l/ml for *F. solani* and 0.035 μ l/ml for *S. parasitica* that was significantly different from MFC of malachite green on *F. solani* (0.300 μ l/ml) and *S. parasitica* (0.120 μ l/ml) (p<0.05).

The results of 48 h and 96 h LC50 is shown in table 3, the results indicated that 48 h and 96 h LC50 are 35.98±0.82ppm. There was not any difference between LC50 after 48, 72 and 96 hours after exposure to combined essential oils and after 48 hours, mortality rate was the same.

Table 1. Physico-chemical parameters of aquarium water in LC50 examination

Physico-chemical parameters		Rate
Temperature (°C)	10±0.5	
Dissolved oxygen (mg/L)	11±1	
Total Hardness (mg/L) $Caco_3$		195±15
PH	8±0.2	

Table 2. Results of MICs and MFCs of malachite green and combined essential oils on *saprolegnia parasitica* and *fusarium solani* (µl/ml)

Fungal strain	drug	MIC ±	SE	MFC±	SE
Fusarium solani	malachite green	0.060±0.001	0.300±	0.010	
Fusarium solani	combined essential oils	0.025±	0.003	0.050±0.002	
Saprolegnia parasitica	malachite green	0.045±	0.001	0.120±0.002	
Saprolegnia parasitica	combined essential oils	0.018±0.0	0.035±0.001		

Table 3. Mean ± standard error of combined essential oils LC50, after 1, 12, 24, 48, 72 and 96 hours on rainbow trout fingerlings

Time (hours)	LC50 (Mean ± SE) (ppm)
1	41.70±1.00
12	35.48±0.80
24	35.22±0.80
48	35.98±0.82
72	35.98±0.82
96	35.98±0.82

Table 4. Toxicity of agents and chemicals based on LC50 48 hours (ppm) (Svobodova and Vikosoa, 1991)

Serial	Toxicity Rate	LC50 Concentration Limits
1	Very Low Toxic	1000<LC50<10000
2	Low Toxic	100<LC50<1000
3	Moderate Toxic	10<LC50<100
4	High Toxic	1<LC50<10
5	Very High Toxic	0.1<LC50<1
6	Very Very Toxic	LC50<0.1

Discussion

The major components of the economically essential oils and herbal extracts are summarized by Bauer *et al.* (2001). Major components can constitute up to 85% of the oils, whereas other components are present only as a trace (Senatore, 1996; Bauer *et al.*, 2001). The most important components of the CEO used in the current study were 1, 8-cineol, thymol, camphor, α-thujone, menthon and menthol. All of these compounds have been shown to have antifungal activities against filamentous fungi in other studies (Cowan, 1999; Mahasneh and El-Oqlah, 1999; Marino *et al.*, 2001; Iscan *et al.*, 2002; Burt, 2004; Pina-Vaz *et al.*, 2004, Mousavi *et al.*, 2009). Their mechanisms of action have been determined and are attributed with disturbance of cell membranes, disrupting the proton motive force, electron flow, active transport and resulting in coagulation of intracellular contents (Burt, 2004). Some researchers supposed that combinations of essential oils have greater antifungal activity than their individual components due to their synergistic effects (Cowan, 1999; Pina-Vaz *et al.*, 2004; Duarte *et al.*, 2005; Lee *et al.*, 2007, Mousavi *et al.*, 2009).

So, in this study, *in vitro* susceptibility of filamentous fungi (*Saprolegnia parasitica* and *Fusarium solani*) against malachite green and the combined essential oils was determined and then LC50 of combined essential oils was determined *in vivo* on rainbow trout fingerlings.

Results of MIC and MFC of the combined essential oils indicated that the combined essential oils can inhibit fungal growth and have a fungicidal effect in lower concentration in comparison with malachite green.

Some studies on the antifungal activities of essential oils and herbal extracts have tested their potential for controlling filamentous fungi (Vidya and Vidya, 2000; Velickovic *et al.*, 2003; Segvic Klaric *et al.*, 2007, Mohsenzadeh, 2007; Rai and Bansod, 2008; Musyimi and Ogur, 2008; Khan *et al.*, 2009; Eghtesad *et al.*, 2009). The Researchers showed that some of herbal extracts have potentially antifungal activities on filamentous fungi, especially *Fusarium solani* and *Saprolegnia parasitica*, but the MICs and MFCs of the combined essential oils which are used in this study are lower than previous reports on individual extracts and they are in agreement with the results reported by some researcher on combination of essential oils and herbal extracts (Duarte *et al.*, 2005; Lee *et al.*, 2007; Al-Bayati, 2007; Mousavi *et al.*, 2009).

In this study, 48 h and 96 h LC50 were 34.98ppm. LC50, 48, 72 and 96 hours was the same and 48 hours after exposure to combined essential oils, mortality rates were fixed. LC50 of some of the chemicals and herbal extracts were reported. 96 h LC50 of formalin (0.072 mg/L),

malachite green (0.035 mg/L) and copper sulphate (3.1-4.4 mg/L, based on water hardness) were determined by Post (1987). In another study, Strivastava *et al* determined 48 h and 96 h LC50 of malachite green on *Heteropneustes fossilis* fingerlings. (1995) and their values were 1.4mg/L and 1mg/L, consequently. Furthermore, Bill *et al* reported that LC50 of malachite green after 6 hours on *Oncorhynchus mykiss* was 1.4 mg/L (Bill *et al.*, 1977).

Hadjikhoondi *et al*. (2000) examined Chemical activity of *Mentha spicata* L. essential oil on *Anophel stephani* and *Artemia salina* and they reported that 24 h LC50 of this essential oil were 9±5μg/ml and 9.2μg/ml respectively.

Abd-Elmageed *et al*. (2008) found that *Salvia officinalis* be potent against brine shrimps with LC50 value of 55.1- 55.6ppm.

Based on the results which were obtained from this study in comparison to table 4, the combined essential oils is an agent with moderate toxicity but malachite green is a chemical agent with high toxicity. Toxicology and teratology effects of malachite green on fish and fish eggs and other animals have been reported (Culp *et al.*, 2006). But there is not any report for toxicity effect of these herbal extract on fish and human up to present. Therefore, the combined essential oils can be a substitute for chemical agents for controlling fungal and mold infection diseases in aquaculture.

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اثرات ضدقارچی و سمیت اسانس ترکیبی جدید در ماهی قزل آلای رنگین کمان در مقایسه با مالاشیت گرین

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چکیده

عفونت قارچی، یکی از مهمترین مشکلات در آبزی پروری است. اخیرا، استفاده از بعضی مواد شیمیایی نظیر مالاشیت گرین به منظور کنترل آلودگی های قارچی در آبزی پروری با محدودیت مواجه شده است. در دهه اخیر، محققین بر استفاده از عصاره های گیاهی و روغن‌های ضروری در آبزی پروری توجه خاصی نموده اند. در این مطالعه، حداقل غلظت مهاری (MIC) و حداقل غلظت کشندگی قارچی (MFC) یک ترکیب جدید از چهار اسانس گیاهی (*Eucalyptus globulus*، *Menthapiperita*، *Salvia officinalis* و *Thymus vulgaris*) توسط روش رقت سازی سریالی تعیین گردید. سپس، غلظت نیمه کشندگی اسانس ترکیبی در ۴۸ ساعت و ۹۶ ساعت بر روی بچه ماهی قزل آلای رنگین کمان مورد ارزیابی قرار گرفت. MIC و MFC اسانس ترکیبی به ترتیب ۰/۰۲۵ میکرولیتر در میلی لیتر و ۰/۰۵۰ میکرولیتر در میلی لیتر برای *فوزاریوم سولانی* و ۰/۰۱۸ میکرولیتر در میلی لیتر و ۰/۰۳۵ میکرلیتر در میلی لیتر برای *ساپرولگنیا پارازیتیکا* بود که کمتر از مقادیر به دست آمده در ارتباط با مالاشیت گرین (۰/۰۶۰ میکرولیتر در میلی لیتر و ۰/۳۰۰ میکرولیتر در میلی لیتر برای *فوزاریوم سولانی* و ۰/۰۴۵ میکرولیتر در میلی لیتر و ۰/۱۲۰ میکرولیتر در میلی لیتر برای *ساپرولگنیا پارازیتیکا*) بود ($p<0.05$). نتایج تعیین غلظت نیمه کشندگی ۴۸ و ۹۶ ساعته برابر با ۳۴/۹۸ بود. بر اساس نتایج بدست آمده، اسانس ترکیبی مورد استفاده در این تحقیق، می‌تواند به عنوان یک ترکیب ضدقارچ در هچری‌ها و مراکز تکثیر ماهیان سردآبی مورد استفاده قرار گیرد.

واژگان کلیدی: عفونت قارچی، هچری‌های آزادماهیان، اسانس گیاهی، MIC، MFC، LC50